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Box Patent Application

Assistant Commissioner for Patents
Washington, DC 20231

Presented for filing is a new continuation-in-part patent application of:

Applicant: SHIGEAKI KATO, KEN-ICHI TAKEYAMA AND SACHIKO KITANAKA

Title: GENE SCREENING METHOD USING NUCLEAR RECEPTOR

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Enclosed are the following papers, including those required to receive a filing date under 37 CFR 1.53(b):

	Pages
Specification	31
Claims	6
Abstract	1
Declaration	[To be Filed at a Later Date]
Drawing(s)	12

Enclosures:
— Postcard.

This application is a continuation-in-part (and claims the benefit of priority under 35 USC 120) of U.S. application serial no. PCT/JP98/03280, filed July 22, 1998. The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

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Basic filing fee	$690
Total claims in excess of 20 times $18	$126
Independent claims in excess of 3 times $78	$468
Fee for multiple dependent claims	$0
Total filing fee:	$1284

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Respectfully submitted,

Janis K. Fraser, Ph.D., J.D.
Reg. No. 34,819

Enclosures

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Attorney's Docket No.: 06501-054001

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: GENE SCREENING METHOD USING NUCLEAR RECEPTOR

APPLICANT: SHIGEAKI KATO, KEN-ICHI TAKEYAMA AND SACHIKO KITANAKA

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GENE SCREENING METHOD USING NUCLEAR RECEPTOR

Cross Reference to Related Applications

This application is a continuation-in-part of International Patent Application No. PCT/JP98/03280, filed July 22, 1998, which claims priority from Japanese Patent Application No. JP 09/212624, filed July 22, 1997.

Technical Field

This invention relates to a method for screening a compound using the nature of transcriptional regulatory factors, mainly nuclear receptors, and a method for determining the compound.

Specifically, it relates to a method for screening a gene encoding a polypeptide that converts a ligand precursor into a ligand, a polypeptide that converts a ligand precursor obtainable by the screening method into a ligand, a gene encoding the polypeptide, and a method for determining whether or not a test gene encodes a polypeptide that converts a ligand precursor into a ligand. In addition, it relates to a method for screening a ligand that binds to a nuclear receptor, a ligand obtainable by the screening method, and a method for determining whether or not a test compound is a ligand that binds to a nuclear receptor. Furthermore, it relates to a method for screening a gene encoding a polypeptide that converts an inactive form of a transcriptional regulatory factor into an active form.

Background of the Invention

1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) (A. W. Norman, J. Roth, L. Orchi, Endocr. Rev. 3, 331 (1982); H. F. DeLuca, Adv. Exp. Med. Biol. 196, 361 (1986); M. R. Walters, Endocr. Rev. 13, 719 (1992)) is a hormone form of vitamin D and the most

biologically active natural metabolite. This compound is generated by sequential hydroxylation. First, it is hydroxylated in the liver to generate 25-hydroxyvitamin D_3 ($25(OH)D_3$), then subsequently hydroxylated in the kidney to generate $1\alpha,25(OH)_2D_3$ (H. Kawashima, S. Torikai, K. Kurokawa, Proc. Natl. Acad. Sci. USA 78, 1199 (1981); H. L. Henry et al., J. Cell. Biochem. 49, 4 (1992)). The transactivation effect of vitamin D receptor (VDR) is provoked by the binding of $1\alpha,25(OH)_2D_3$ to VDR (M. Beato, P. Herrlich, G. Schuts, Cell 83, 851 (1995); H. Darwish and H. F. DeLuca, Eukaryotic Gene Exp. 3, 89 (1993); B. D. Lemon, J. D. Fondell, L. P. Freedman, Mol. Cell. Biol. 17, 1923 (1997)). This regulates the transcription of a series of target genes involved in the major functions of vitamin D, such as calcium homeostasis, cell differentiation, and cell proliferation (D. D. Bikle and S. Pillai, Endoc. Rev. 14, 3 (1992); R. Bouillon, W. H. Okamura, A. W. Norman, Endoc. Rev. 16, 200 (1995); M. T. Haussler et al., Recent Prog. Horm. Res. 44, 263 (1988); P. J. Malloy et al., J. Clin. Invest. 86, 2071 (1990)). The importance of the hydroxylation of $25(OH)D_3$ in the kidney in the synthesis of active vitamin D has been shown, and it has been believed for a long time that the hydroxylation is done by $25(OH)D_3$-1α hydroxylase (1α(OH)-ase), which is localized especially at proximal renal tubules. The activity of 1α(OH)-ase is negatively regulated by its final product, $1\alpha,25(OH)_2D_3$ (Y. Tanaka and H. F. DeLuca, Science 183, 1198 (1974); K. Ikeda, T. Shinki, A. Yamaguchi, H. F. DeLuca, K. Kurokawa, T. Suda, Proc. Natl. Acad. Sci. USA 92, 6112 (1995); H. L. Henry, R. J. Midgett, A. W. Norman, J. Biol. Chem. 249, 7584 (1974)), and positively regulated by peptide hormones like calcitonin and PTH, which participate in calcium regulation (H. Kawashima, S. Torikai, K. Kurokawa, Nature 291, 327 (1981); K. W. Colston, L. M. Evans, L. Galauto, L. Macintyre, D. W. Moss, Biochem. J. 134, 817 (1973); D. R. Fraser and E. Kodicek, Nature 241, 163 (1973); M. J. Beckman, J. A. Johnson, J. P. Goff, T. A. Reinhardt, D. C.

Beitz, R. L. Horst, Arch. Biochem. Biophys. 319, 535 (1995)). The complicated regulation of the 1α (OH)-ase activity by these hormones maintains the serum concentration of $1\alpha,25(OH)_2D_3$ at a certain level. The mutation of the 1α(OH)-ase gene may causes a genetic disease, vitamin D-dependent type I rickets (D. Fraser, S. W. Kooh, H. P. Kind, M. F. Hollick, Y. Tanaka, H. F. DeLuca, N. Engl. J. Med. 289, 817 (1973); S. Balsan, in Rickets, F. H. Glorieux, Ed. (Raven, New York, 1991), pp 155-165), which also demonstrate the importance of the enzyme *in vivo* in the function of vitamin D. The biochemical analysis of partially purified 1α(OH)-ase protein strongly suggested that this enzyme belongs to P450 family (S. Wakino et al., Gerontology 42, 67 (1996); Eva Axen, FEBS Lett. 375, 277 (1995); M. BurgosTrinidad, R. Ismaii, R. A. Ettinger, J. M. Prahl, H. F. DeLuca, J. Biol. Chem. 267, 3498 (1992); M. Warner et al., J. Biol. Chem. 257, 12995 (1982)). However, the biochemical characteristics of the enzyme and the molecular mechanism of the negative feedback by $1\alpha,25(OH)_2D_3$ are not well understood since the enzyme purification is difficult and cDNA has not been cloned yet. Thus, the cDNA cloning of the enzyme had been desired. Recently, the cloning of the rat enzyme that hydroxylates the 1α position of vitamin D has been reported (J. Bone Min. Res. Vol. 11 (suppl) 117 (1996)).

Conventionally, methods depend on phosphorylation of intracellular signal transduction factors or ion channels of membrane receptors as criteria have mainly been employed to screen genes encoding polypeptides that act on a specific nuclear receptor directly or indirectly, including 1α (OH)-ase mentioned above. Specifically, expression vectors into which a cDNA library or cDNA is inserted are introduced into cells or haploid individuals, for example *Xenopus* oocytes, and then phosphorylation, cell growth and the change in the electric current has been monitored for the screening.

However, it has been very difficult to isolate genes by using these methods. Especially, highly sophisticated techniques are required for the expression cloning of an enzyme itself because the indicators available for the detection are limited. Therefore, the development of a simple and efficient screening method has been desired.

Summary of the Invention

An objective of the present invention is to provide a simple and efficient method for screening a gene encoding a polypeptide that converts a ligand precursor into a ligand, and a method for determining whether or not a test gene encodes a polypeptide that converts a ligand precursor into a ligand. Another objective of the present invention is to provide a method for isolating a polypeptide that converts a ligand precursor into a ligand and a gene encoding it, using the screening method.

Furthermore, an objective of the invention is to provide a method for screening a ligand that binds to a nuclear receptor, a method for determining whether or not a test compound is a ligand for a nuclear receptor, and a method for screening a gene encoding a polypeptide that converts an inactive form of a transcriptional regulatory factor into an active form, based on the screening method and the determination method described above.

The present inventors investigated to achieve the above objectives and focused on the nature of nuclear receptors, which function as transcriptional regulatory factor by being bound by a ligand. We successfully constructed the system in which a ligand is formed by the expression of a polypeptide that converts a ligand precursor into a ligand, and the ligand thus formed binds to a nuclear receptor to thereby induce the expression of a reporter gene located downstream of the target sequence. We searched a gene library using this system and succeeded in isolating a gene encoding a polypeptide capable of converting a ligand precursor into a ligand.

Specifically, the inventors constructed a vector comprising a gene encoding a fusion polypeptide of DNA binding domain of GAL4 and ligand-binding domain of vitamin D receptor and a vector in which the lacZ gene, a reporter, is located downstream of the binding sequence of the DNA binding domain of GAL4. These two vectors, and subsequently the cDNA library, were introduced into cells. Then the reporter activity was measured after adding the vitamin D precursor. Clones with the reporter activity were selected to examine whether or not they have the activities to convert the vitamin D precursor into vitamin D, thereby finding a clone that has the activity.

Also, the inventors found that this system, which takes the advantage of the transcriptional regulatory function of a nuclear receptor, makes it possible to screen a ligand that binds to a nuclear receptor and to examine whether or not a test compound is a ligand that binds to the nuclear receptor. Specifically, for example, a library of test compounds can be used in place of a ligand precursor and a gene library comprising the gene encoding a polypeptide that converts a precursor into a ligand in the system described above. When a test compound functions as a ligand, the nuclear receptor promotes the transcription of the reporter gene. Thus, compounds that function as ligands can be screened from the library simply by detecting the reporter activity as an index.

Furthermore, the inventors found that the system utilizing the transcriptional regulatory function of a nuclear receptor can be employed to screen genes that encode polypeptides capable of converting an inactive form of a wide range of transcriptional regulatory factors into an active form. In other words, the inventors found that the system in which the transcriptional regulatory function can be used to isolate factors involved in activation of various transcriptional regulatory factors, which have inactive and active forms,

such as transcriptional regulatory factors activated by phosphorylation as well as nuclear receptors activated by the binding of ligands.

More specifically, this invention relates to:

1. a cell comprising a vector carrying a gene encoding a nuclear receptor and a vector carrying the binding sequence of the nuclear receptor and a reporter gene located downstream of said binding sequence;

2. the cell of 1, wherein the nuclear receptor is a vitamin D receptor;

3. a cell comprising a vector carrying a gene encoding a fusion polypeptide comprising DNA binding domain of a nuclear receptor and ligand-binding domain of a nuclear receptor, and a vector carrying the binding sequence of the DNA binding domain of the nuclear receptor and a reporter gene located downstream of the binding sequence;

4. the cell of 3, wherein the DNA binding domain of the nuclear receptor is originated from GAL4;

5. the cell of 3, wherein the ligand-binding domain of the nuclear receptor is originated from vitamin D receptor;

6. a method for screening a ligand that binds to a nuclear receptor, the method comprising

(A) contacting a test compound with the cell of any one of 1 to 5,

(B) detecting the reporter activity, and

(C) selecting the test compound which elicited the reporter activity in the cell;

7. a method for determining whether or not a test compound is a ligand that binds to a nuclear receptor, the method comprising,

(A) contacting a test compound with any one of the cell of 1 to 5, and

(B) detecting the reporter activity;

8. a method for screening a gene encoding a polypeptide that converts a ligand precursor into a ligand, the method comprising

(A) introducing a test gene into any one of the cell of 1 to 5,

(B) contacting a ligand precursor to the cell into which the test gene is introduced,

(C) detecting the reporter activity, and

(D) isolating the test gene from the cell which showed the reporter activity;

9. a method for determining whether or not a test gene encoding a polypeptide that converts a ligand precursor into a ligand, the method comprising

(A) introducing a test gene into any one of the cell of 1 to 5,

(B) contacting a ligand precursor to the cell into which the test gene is introduced, and

(C) detecting the reporter activity;

10. a method for screening a gene encoding a polypeptide that converts an inactive form of vitamin D_3 into an active form, the method comprising

(A) introducing a test gene into the cell of 2 or 5,

(B) contacting an inactive form of vitamin D_3 to the cell into which the test gene is introduced,

(C) detecting the reporter activity, and

(D) isolating the test gene from the cell that shows the reporter activity;

11. a method for determining whether or not a test gene encodes a polypeptide that converts an inactive form of vitamin D_3 into an active form, the method comprising

(A) introducing a test gene into the cell of 2 or 5,

(B) contacting an inactive form of vitamin D_3 with the cell into which the test gene is introduced, and

(C) detecting the reporter activity;

12. a ligand that binds to a nuclear receptor, which is obtainable by the method of claim 6;

13. a gene encoding a polypeptide that converts a ligand precursor into a ligand, which is obtainable by the method of claim 8.

14. a gene encoding a polypeptide that converts an inactive form of vitamin D_3 into an active form, which is obtainable by the method of claim 10.

15. a polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or its derivative comprising said sequence in which one or more amino acids are substituted, deleted, or added, and having activity to convert an inactive form of vitamin D_3 into an active form;

16. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or its derivative comprising said sequence in which one or more amino acids are substituted, deleted, or added, and having activity to convert an inactive form of vitamin D_3 into an active form;

17. a polypeptide encoded by a DNA that hybridizes with a DNA having the nucleotide sequence of SEQ ID NO: 3, wherein the polypeptide has activity to convert an inactive form of vitamin D_3 into an active form;

18. a polypeptide encoded by a DNA that hybridizes with the nucleotide sequence of SEQ ID NO: 4, wherein the polypeptide has activity to convert an inactive form of vitamin D_3 into an active form;

19. a DNA encoding any one of the polypeptide of 15 to 18;

20. a DNA hybridizing with a DNA having the nucleotide sequence of SEQ ID NO: 3 and encoding a polypeptide having activity to convert an inactive form of vitamin D_3 into an active form;

21. a DNA hybridizing with a DNA having the nucleotide sequence of SEQ ID NO: 4 and encoding a polypeptide having activity to convert an inactive form of vitamin D_3 into an active form;

22. a vector comprising any one of the DNA of 19 to 21;

23. a transformant expressively retaining any one of the DNA of 19 to 21;

24. a method for producing any one of the polypeptide of 15 to 18, the method comprising culturing the transformant of 23;

25. an antibody that binds to any one of the polypeptide of 15 to 18;

26. a method for screening a gene encoding a polypeptide that converts an inactive form of transcriptional regulatory factor into an active form, the method comprising

(A) introducing a test gene into cells into which a vector comprising a gene encoding an inactive form of transcriptional regulatory factor and a vector comprising the binding sequence of said inactive transcriptional regulatory factor and a reporter gene located downstream thereof are introduced,

(B) detecting the reporter activity, and

(C) isolating the test gene from the cells showing the reporter activity;

27. a method of 26, wherein the inactive transcriptional regulatory factor is a complex of non-phosphorylated NFκB and IκB, non-phosphorylated HSTF, or non-phosphorylated AP1.

The term "ligand" used herein means a compound that binds to a nuclear receptor and regulates the transcriptional activating ability of a target gene of the nuclear receptor. It includes not only naturally-occurring compounds but also synthetic compounds.

The term "nuclear receptor" used herein means a factor that binds to an upstream site of a promoter of a target gene and ligand-dependently regulates transcription.

The "polypeptide that converts a ligand precursor into a ligand" includes a polypeptide that acts directly on a ligand precursor to convert it into a ligand. It also includes a polypeptide that indirectly converts a ligand precursor into a ligand, for example, a polypeptide activating a polypeptide that directly acts on a ligand precursor to convert it into a ligand.

An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in mixtures of different (i) DNA molecules, (ii) transfected cells, or (iii) cell clones: e.g., as these occur in a DNA library such as a cDNA or genomic DNA library.

The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological macromolecules. The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry weight. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

The "transcriptional regulatory factor" used herein means a factor that binds to an upstream site of a promoter of a target gene and regulates transcription of the target gene. The above-described nuclear receptor is included in the transcriptional regulatory factor of the invention.

The "polypeptide that converts an inactive form of transcriptional regulatory factor into an active form" used herein includes not only a polypeptide that acts directly on an inactive form of transcriptional regulatory factor to convert it into an active form but also a polypeptide that indirectly converts an inactive form to an active form. When an inactive form of transcriptional regulatory factor is converted into an active form by phosphorylation, the transcriptional regulatory factor of the invention includes a polypeptide that activates a polypeptide phosphorylating the inactive form and indirectly converts the inactive form into the active form as well as a polypeptide directly involved in the phosphorylation.

The first aspect of the present invention relates to a method for screening a gene encoding a polypeptide that converts a ligand precursor into a ligand, and a method for determining whether or not a test gene encodes a polypeptide that converts a ligand precursor into a ligand. In these methods, a vector carrying a gene encoding a nuclear receptor (expression unit 1), and a vector carrying the binding sequence of the nuclear receptor and a reporter gene located downstream thereof (expression unit 2) are introduced into cells. Then, a test gene is introduced into the cells.

The "gene encoding a nuclear receptor" in the expression unit 1 is not particularly limited and any nuclear receptor gene can be used. For example, when orphan receptors such as PPAR, LXR, FXR, MB67, ONR, NUR, COUP, TR2, HNF4, ROR, Rev-erb, ERR, Ftz-F1, Tlx and GCNF (Tanpakusitsu Kakusan Koso (Protein, Nucleic Acid, Enzyme) Vol. 41 No. 8 p1265-1272 (1996)) are used as the nuclear receptor in the below-mentioned screening of unknown ligands that bind to nuclear receptors or determination whether or not a test compound is a ligand binding to a nuclear receptor, the naturally-occurring or synthesized ligand can be detected and isolated. Furthermore, nuclear receptors for which the ligand and ligand precursor are known, such as VDR (vitamin D receptor), ER, AR, GR, MR (Tanpakusitsu Kakusan Koso (Protein, Nucleic Acid, Enzyme) Vol. 41 No. 8 p1265-1272 (1996)) are preferably used in the below-mentioned screening of genes encoding polypeptides that convert a ligand precursor into a ligand or the determination whether or not a test gene encodes a polypeptide that converts a ligand precursor into a ligand. However, nuclear receptors used in the present invention are not limited thereto.

In the present invention, the nuclear receptor gene can be used alone, and a fusion polypeptide gene comprising the DNA binding domain of a nuclear receptor and the ligand-binding domain of another nuclear receptor can also be used. For example, the DNA binding domain of GAL4 is preferably used as the DNA binding domain because it enhances the expression of the reporter gene downstream thereof.

The "binding sequence of a nuclear receptor" in the expression unit 2 varies depending on the nuclear receptor. In most nuclear receptors, sequences comprising "AGGTCA" are usually used. In the case of a dimeric nuclear receptor, the binding sequence is preferably composed of two repetition of the sequence. The repetitive sequences include the direct-repeat type, in which the two sequences are aligned in the same direction,

and the palindrome type, in which the sequences are directed to the center (Tanpakusitsu Kakusan Koso (Protein, Nucleic Acid, Enzyme) Vol. 41 No. 8 p1265-1272 (1996)). A spacer sequence usually exists between the repetition sequences, which can determine the specificity of the nuclear receptor (K. Umesono et al., Cell Vol. 65, p1255-1266 (1991)).

A reporter gene located downstream of a nuclear receptor is not particularly limited. Preferable reporter genes are, for example, lacZ, CAT, and luciferase. Resistant genes to toxins or antibiotics, such as ampicillin resistant gene, tetracycline resistant gene, kanamycin resistant gene, can also be used to select cells by applying the corresponding toxin or antibiotic.

The binding sequence of a nuclear receptor and the reporter gene are not necessarily connected directly. Some sequences that alter the strength of the promoter, for example, the promoter region of -globin, can be inserted between the binding sequence and the reporter gene.

Animal cells are preferable for introducing these expression units. Cells with high transformation efficiency such as COS-1 cells and HeLa cells are particularly preferable. Vectors for animal cells such as "pcDNA3" (Invitrogen) are preferred to construct expression units. Vectors can be introduced into host cells by a known method such as calcium phosphate method, lipofection method, electroporation method and the like.

A test gene is introduced into cells thus prepared. A test gene is not particularly limited, and any genes whose capability of converting a ligand precursor into a ligand is detected can be used. Genes are screened from cells or cDNA libraries prepared from mRNA isolated from tissues or the like, which are expected to express an objective gene. For example, a gene encoding a polypeptide that converts vitamin D precursor into active vitamin D can be screened from a cDNA library derived from kidney or the like. In this case,

a vector expressing adrenodoxin (ADX) and an vector expressing adrenodoxin reductase (ADR) are preferably introduced into cells together with a test gene so as to efficiently generate active vitamin D. A test gene can be inserted into an appropriate vector and introduced into cells. For example, preferable vectors are 'pcDNA3' (Invitrogen) mentioned above or the like.

Next, cells into which a test gene is introduced are contacted with a ligand precursor. As the ligand precursor, the one that acts on a nuclear receptor expressed by the expression unit 1 mentioned above is usually used. Examples of the ligand precursor include, without limitation, 25-hydroxyvitamin D_3, a precursor of VDR ligand (active vitamin D, $1\alpha,25(OH)_2D_3$); testosterone, a precursor of ER ligand (estrogen) and AR ligand (dihydroxytestosterone); 11-deoxycortisol, a precursor of GR ligand (cortisol), corticosterone, a precursor of MR ligand (aldosterone), etc. The contact of the ligand precursor with the cells can be performed by adding the ligand precursor to the culture medium of the cells, or a similar method.

The reporter activity is then detected. If a test gene that is introduced into cells encodes a polypeptide that converts a ligand precursor into a ligand, the ligand generates from the ligand precursor contacted with the cells, and binds to the nuclear receptor to make a ligand- nuclear receptor complex, which then binds to its target sequence to express the reporter gene. If the test gene does not encode a polypeptide that converts a ligand precursor into a ligand, the ligand is not produced from the ligand precursor and thus the reporter gene is not expressed. In this way, detecting the reporter activity enables judging whether or not the test gene encodes a polypeptide that converts a ligand precursor into a ligand. The reporter activity can be detected by a method well known in the art using criteria such as staining, fluorescence, or cell viability, depending on the reporter gene.

When a gene library or the like is used instead of a single gene, cells are selected by the reporter activity to isolate the test gene. The test gene can be extracted from cells by, for example, the method described in H. S. Tong et al., Journal of Bone and Mineral Research Vol. 9, 577-584 (1994). The primary structure of the gene extracted can be determined by a known method such as dideoxy method.

The cells into which expression units 1 and 2 are introduced can be used for screening genes encoding polypeptides capable of converting a ligand precursor into a ligand or determining whether or not a test gene encodes a polypeptide that converts a ligand precursor into a ligand. Furthermore, the cells can be used for screening ligands that bind to a nuclear receptor or determining whether or not a test compound is a ligand that binds to a nuclear receptor. Specifically, a candidate for a ligand that acts on a nuclear receptor (a single test compound or a library of test compounds) is used instead of a ligand precursor and a candidate for a gene encoding a polypeptide that converts the ligand precursor into the ligand (a single candidate gene, gene libraries, etc.). When a test compound functions as a ligand, a complex of a nuclear receptor and the test compound (ligand) activates the reporter located downstream of the target sequence and thus whether or not the test compound function as a ligand can be judged. Furthermore, compounds that function as ligands can be screened from plural compounds by detecting the reporter activity.

The inventors screened genes encoding polypeptides capable of converting the vitamin D precursor into active vitamin D as an example of the screening of genes encoding enzymes capable of converting a ligand precursor into a ligand, and obtained a desired gene. The present invention also relates to a polypeptide that converts the vitamin D precursor into active vitamin D and a gene encoding it.

Polypeptides derived from mouse and human that convert the vitamin D precursor into active vitamin D, which are encompassed by the polypeptides of the present invention, are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. Vitamin D is first hydroxylated in the liver to generate $25(OH)D_3$, then hydroxylated in the kidney to generate $1\alpha,25(OH)_2D_3$. The polypeptide of the present invention converts $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$ by hydroxylation, namely hydroxylates the 1α position of vitamin D (1α (OH)-ase).

The polypeptide of the present invention can be a naturally-occurring protein. Alternatively, it can be prepared as a recombinant polypeptide by gene recombination techniques. Both are included in the polypeptide of the present invention. A naturally-occurring protein can be isolated by methods well known in the art, for example, from kidney cell extract by affinity chromatography using an antibody binding to the polypeptide of the present invention as described below. On the other hand, a recombinant protein can be prepared by culturing cells transformed with a DNA encoding the polypeptide of the present invention as described below.

In addition, those skilled in the art can prepare polypeptides with substantially the same biological activity as the polypeptide set forth in SEQ ID NO: 1 (or SEQ ID NO: 2) by substituting amino acid(s) of the polypeptide or the like known method. The mutation of amino acids can occur spontaneously. The polypeptide of the present invention also includes the mutants of the polypeptide set forth in SEQ ID NO: 1 (or SEQ ID NO: 2) whose amino acid(s) are modified by substitution, deletion or addition, and which possesses the activity to convert the inactive form of vitamin D_3 into the active form. A "conservative amino acid substitution" is one in which an amino acid residue is replaced with another residue having a chemically similar side chain. Families of amino acid residues having similar side chains

have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). The known method of modifying an amino acid sequence is, for example, the method described in the literature, "Shin Saiboukougaku Jikken Protocol, Ed. Department of Oncology, The Institute of Medical Science, The University of Tokyo, p241-248." Mutations can be introduced by using commercially available 'QuickChange Site-Directed Mutagenesis Kit' (Stratagene).

It is a routine for those skilled in the art to prepare probes based on the entire or the partial nucleotide sequence of SEQ ID NO: 3 encoding the mouse polypeptide or SEQ ID NO: 4 encoding the human polypeptide, isolate DNAs with high homology with the probes from other species, and obtain polypeptides having the activities substantially equivalent to those of the polypeptide of the present invention using a known method such as hybridization technique (K. Ebihara et al., Molecular and Cellular Biology, Vol. 9, 577-584 (1994)) or polymerase chain reaction technique (S. Kitanaka et al., Journal of Clinical Endocrinology and Metabolism, Vol. 82, 4054-4058 (1997)). Therefore, the polypeptides of the present invention include those encoded by DNAs that hybridize under stringent conditions with the DNA having the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 4, and having the activity to convert an inactive form of vitamin D_3 into an active form. By "stringent conditions" is meant hybridization at 37°C, 1 X SSC, followed by washing at 42°C, 0.5 X SSC. Animal species used for isolating DNAs hybridizing with the DNA having the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 4 include rat, monkey, etc. DNAs

encoding polypeptides with biological activities substantially equivalent to those of the polypeptide set forth in SEQ ID NO: 1 or SEQ ID NO: 2 usually have high homology with the DNA set forth in SEQ ID NO: 3 or SEQ ID NO: 4. The "high homology" means sequence identity of 70% or more, preferably 80% or more, and more preferably 90% or more. The "percent identity" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3. Where gaps exist between two sequences, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See http://www.ncbi.nlm.nih.gov.

Another aspect of the present invention relates to a DNA encoding the polypeptide of the present invention described above. The DNA of the present invention can be cDNA, genomic DNA, or synthetic DNA. It can be used not only to isolate a polypeptide with activities substantially equivalent to those of the polypeptide of the present invention from other species, but also to produce the polypeptide of the present invention as a recombinant polypeptide. Specifically, the DNA encoding the polypeptide of the present invention, for example, the DNA set forth in SEQ ID NO: 3 or SEQ ID NO: 4, is inserted into an appropriate vector, which are introduced into appropriate cells. The transformant cells are

cultured to express the polypeptide, and the recombinant polypeptide is purified from the culture.

The cells used to produce the recombinant polypeptide include, for example, *Escherichia coli* and mammalian cells. The vectors used for expressing the recombinant polypeptide in the cells vary depending on host cells. For example, pGEX (Pharmacia) and pET (Novagen) are suitably used for *E. coli*, and pcDNA3 (Invitrogen) is used suitably for animal cells. These vectors can be introduced into the host cells by heat-shock, for example. The recombinant polypeptide can easily be purified from the transformant by glutathione-Sepharose affinity chromatography when pGEX (Pharmacia) is used, and by nickel-agarose affinity chromatography when pET (Novagen) is used.

Those skilled in the art can readily raise antibodies that bind to the polypeptide of the invention using the polypeptide prepared as described above. The polyclonal antibodies of present invention can be prepared by a well known method. For example, the polypeptide is injected into a rabbit or the like and IG fraction is purified by ammonium sulfate precipitation. Monoclonal antibodies can be produced by preparing hybridoma from spleen cells of mice immunized with the polypeptide of the present invention and myeloma cells and culturing the hybridoma to secrete the monoclonal antibody in the culture medium, intraperitoneally injecting the antibody obtained into an animal to obtain a large quantity of the antibody.

The polypeptides, DNA, and antibodies of the present invention can be applied as follows. The polypeptides and DNA of the present invention can be used for therapy and/or diagnosis of patients with low 1α (OH)-ase activity, such as patients with defects in 1α (OH)-ase or renal failure. The present inventors have identified the mutation of the DNA of the present invention in vitamin D-dependent type I rickets case, specifically, P382S

(mutation from CCT to TCT), R335P (mutation from CGG to CCG), G125E (mutation from GGA to GAA), R107H (mutation from CGC to CAC). The present invention is also applicable to treat these patients. The mutations in the patients can be identified by extracting DNA from peripheral leukocytes of a patient, amplifying the DNA by PCR using the primer in which each exon is set as intron, and determining the nucleotide sequence or the DNA by direct sequencing method. The DNA of the present invention can be used in gene therapy. In this case, the DNA of the invention is inserted into an appropriate vector, and the vector is introduced into the body *in vivo* or *ex vivo*, using retrovirus method, liposome method, or adenovirus method. The polypeptides of present invention can be used as an immobilized enzyme to produce active vitamin D derivatives, that is, hydroxylate 1α position of vitamin D or its derivatives without a hydroxyl group at 1α position. Furthermore, the antibodies of the present invention can be used for therapy of such as vitamin D excessiveness, granulomatous diseases, and lymphoma as well as purification of the polypeptides of present invention.

The inventors also enabled screening genes encoding a polypeptide capable of converting an inactive form of various transcriptional regulatory factors into an active form using the above-described screening system of ligands binding to nuclear receptors. Therefore, the present invention also relates to a method for screening a gene encoding a polypeptide that converts an inactive form of a transcriptional regulatory factor into an active form.

There are several reports on the mechanism of the conversion of a transcriptional regulatory factor into its active form. For example, NFκB, a tissue specific factor, is bound to a factor named IκB in the cytoplasm. When it is treated with TPA, IκB dissociates, and NFκB translocates into a nucleus. Considering the effect of TPA treatment, the

phosphorylation by protein kinase C is probably involved in the conversion of NFκB into an active form. In the case of HSTF, its phosphorylation level is low before the heat-shock, and is high after the heat-shock. This indicates that the phosphorylation is involved in the conversion of HSTF into its active form. Phosphorylation is also considered to be involved in the conversion of AP1 into its active form.

GAL4 is an inactive form when GAL80 binds thereto before the induction by galactose. After the induction by galactose, the complex dissociates and GAL4 becomes an active form. Hsp90 binds to a glucocorticoid receptor before the hormone induction. After the induction, the complex dissociate to form an active form of glucocorticoid receptor (Jikken Igaku (Experimental Medicine) Vol. 7, No.4 (1989)).

The "polypeptides that convert an inactive form of a transcriptional regulatory factor into an active form" used herein includes polypeptides functioning in activation of transcriptional regulatory factors by dissociation of inhibitory factors, or by its qualitative alteration, such as phosphorylation. The "inactive form of a transcriptional regulatory factor" include, for example, a complex of non-phosphorylated NFκB and IκB, non-phosphorylated HSTF, non-phosphorylated AP1, as described above, but is not limited thereto.

In this screening method, a gene encoding an inactive form of a transcriptional regulatory factor, instead of a nuclear receptor gene, is introduced into a vector to construct the "expression unit 1" described above, and a vector into which the binding sequence of the transcriptional regulatory factor and a reporter gene downstream thereof is constructed as the "expression unit 2." The expression units are introduced into cells, and a test gene is introduced into the cells. If the test gene introduced has activity to convert an inactive form of the transcriptional regulatory factor into an active form, the inactive transcriptional regulatory factor, which is the product of the expression unit 1, is converted into the active

form, and then active transcriptional regulatory factor binds to its binding sequence in the expression unit 2 to induce expression of the reporter gene. In contrast, when the test gene introduced does not have activity to convert an inactive transcriptional regulatory factor into an active form, the reporter gene in the expression unit 2 will not be induced. Therefore, one can judge whether or not a test gene has activity to convert an inactive transcriptional regulatory factor into its active form using the present screening method by detecting the reporter activity.

When a gene library is used as a test gene, one can isolate a gene encoding a polypeptide with the activity to convert an inactive form of transcriptional regulatory factor into an active form from the library.

Brief Description of Drawings

Figure 1 schematically shows the expression cloning system mediated by VDR.

Figure 2 is a graph showing the serum concentration of $1\alpha,25(OH)_2D_3$ in 3- and 7-week-old VDR+/+, VDR+/- and VDR-/- mice.

Figure 3 is a micrograph of cells stained with X-gal. (b) presents COS-1 cells transformed with a expression cDNA library; (a), negative control; (c), positive control; and (d) stained cells with cDNA that was extracted from the positive cells in (b) and amplified by PCR.

Figure 4 shows the putative amino acid sequence of CYP1AD. The first methionine is assigned as position 1. Asterisk indicates the terminal codon. Putative mitochondria targeting signal is surrounded by square. Underline indicates sterol binding domain. Dotted underline indicates hem-binding domain.

Figure 5 shows homology of 'CYP1AD' to rat 25(OH)-ase (CYP27) and mouse 24(OH)-ase (CYP24). Amino acid sequence homologies in sterol binding domain and hem-binding domain are also indicated.

Figure 6 shows a photograph of 10% SDS-PAGE pattern of CYP1AD protein translated *in vitro*.

Figure 7 shows the result of CAT assay for detecting *in vivo* activity of CYP1AD. The bottom panel shows a representative CAT assay, and the top panel shows the relative CAT activity as average and SEM from three independent experiments.

Figure 8 shows the normal phase HPLC analysis of 25(OH)D_3 metabolites.

Figure 9 shows the reverse phase HPLC analysis of 25(OH)D_3 metabolites.

Figure 10 shows the northern blot analysis for analyzing tissue distribution of CYP1AD transcripts.

Figure 11 shows the northern blot analysis of 3- and 7-week-old, VDR+/+, VDR+/- and VDR-/- mice, with(+) or without(-) overdosage of 1α,25$(OH)_2D_3$ (50 ng/mouse).

Figure 12 shows the relative amount of the hydroxylase gene in 3- or 7-week-old, VDR+/+, VDR+/- and VDR-/- mice, with(+) or without(-) overdosage of 1α,25$(OH)_2D_3$ (50 ng/mouse).

Detailed Description

The present invention is demonstrated with reference to examples below, but is not to be construed being limited thereto.

Example 1

Isolation of cDNA encoding an enzyme that hydroxylates 1α position of vitamin D

The inventors developed an expression cloning system mediated by a nuclear receptor for cloning a full-length cDNA encoding 1α (OH)-ase. The system is based on the mechanism that $25(OH)D_3$, a precursor of $1\alpha,25(OH)_2D_3$, can activate the transactivating function of VDR only in the presence of 1α (OH)-ase (Figure 1). In other words, the ligand-dependent transactivating function of VDR (AF-2) is induced by $1\alpha,25(OH)_2D_3$, but not by $25(OH)D_3$. $25(OH)D_3$ is converted into $1,25(OH)_2D_3$ only in cells expressing 1α (OH)-ase. Therefore, the cells can be detected by X-gal staining (M. A. Frederick et al., Current Protocols in Molecular Biology (Wiley, New York, 1995)) as the result of the expression of the lacZ reporter gene in the presence of $25(OH)D_3$.

In the kidney of 7-week-old VDR-deficient mice (VDR-/- mice), the serum concentration of $1\alpha,25(OH)_2D_3$ was extremely high (Figure 2), which suggested the high 1α (OH)-ase activity. Therefore, the kidney of 7-week-old VDR-/- mice was used to prepare an expression library. Poly(A)$^+$ RNA was purified (K. Takeyama et al., Biochem. Biophys. Res. Commun. 222, 395 (1996); H. Mano et al., J. Biol. Chem. 269, 1591 (1994)), and total cDNA was prepared from poly(A)$^+$ RNA (U. Gubler and B. J. Hoffman, Gene 25, 263 (1983); M. Kobori and H. Nojima, Nucleic Acid Res. 21, 2782 (1993)). The total cDNA was inserted into the HindIII position of pcDNA3 (Invitrogen), a expression vector that is derived from SV40, functions in mammals, and autonomously replicates in COS-1 cells. The reporter plasmid, 17M2-G-lacZ, was constructed by inserting yeast GAL4 (UAS) x 2 and β-globulin promoter into the multicloning site of Basic expression vector (Clontech). The function of AF-2 induced by a ligand was detected using VDR-ligand-binding domain fused

with GAL4-DNA binding domain (VDR-DEF) [GAL4-VDR(DEF)] (K. Ebihara et al., Mol. Cell. Biol. 16, 3393 (1996); T. Imai et al., Biochem. Biophys. Res. Commun. 233, 765 (1997)). Cos-1 cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum were transiently transformed with 0.5 g of GAL4-VDR (DEF) expression vector, 1 g of 17M2-G-lacZ, 0.2 g each of ADX expression vector and ADR expression vector (T. Sakaki, S. Kominami, K. Hayashi, M. AkiyoshiShibata, Y. Yabusaki, J. Biol. Chem. 271, 26209 (1996); F. J. Dilworth et al., J. Biol. Chem. 270, 16766 (1995)), and 0.1 g of the expression cDNA library, using Lipofectin (GIBCO BRL). 10^{-8}M $25(OH)D_3$ was added to the culture medium 12 hours after the transformation. Cells were fixed with 0.05% glutaraldehyde 48 hours after the transformation and were then incubated with X-gal at 37°C for 4 hours to identify β-galactosidase positive cells expressing 1α (OH)-ase by X-gal staining (Figure 3(c)) (M. A. Fredrick et al., Current Protocols in Molecular Biology (Wiley, New York, 1995)). In the negative control, the expression cDNA library was not used (Figure 3(a)). In the positive controls, the expression library was not used, and $1\alpha,25(OH)_2D_3$ was used instead of $25(OH)D_3$ (Figure 3(b)).

The stained cells were selectively collected by micromanipulation using a micropipette with 40 μm diameter under an inverted microscope (H. S. Tong et al., J. Bone Miner. Res. 9, 577 (1994)), then transferred into PCR buffer solution. The PCR products were electrophoresed on 1% agarose gel, and fragments of about 2.0 to 2.5 kb, which is the expected cDNA size of the full-length 1 (OH)-ase, are purified and subcloned into pcDNA3. Sequence analysis of cDNA isolated from randomly selected 64 clones showed that 13 clones encode completely identical ORF. COS-1 cells into which the single cDNA clone was introduced were positive in X-gal staining (Figure 3(d)).

The full-length cDNA was obtained by the colony hybridization screening of the same library using the cDNA as a probe. The amino acid sequence deduced from ORF is a novel polypeptide with 507 amino acids (Figure 4).

The polypeptide, hereinafter called "CYP1AD," has a mitochondria-targeting signal and has significant homologies with P450 family members (D. W. Nebert, DNA Cell. Biol. 10, 1 (1991)). Especially, the homology with rat vitamin D_3 25-hydroxylase is 41.7% and that with mouse 25(OH)D_3 24-hydroxylase is 31.6% (Figure 5)(O. Masumoto, Y. Ohyama, K. Okuda, J. Biol. Chem. 263, 14256 (1988); E. Usui, M. Noshiro, Y. Ohyama, K. Okuda, FEBS Lett. 262, 367 (1990); Y. Ohyama and K. Okuda, J. Biol. Chem. 266, 8690 (1991); S. Itoh et al., Biochem. Biophys. Acta. 1264, 26 (1995)). The homologies for sterol domain, especially conserved domain, in these enzymes are 93% and 60%, respectively, and those for hem binding domain are 70% and 80%, respectively.

The 10% SDS-PAGE analysis of CYP1AD protein, which was translated *in vitro* in the presence of [^{35}S] methionine using Reticulocyte Lysate System (Promega) (H. Sasaki et al., Biochemistry 34, 370 (1995)) revealed that the molecular weight of the polypeptide is approximately 55 kDa (Figure 6), which is identical to the molecular weight of partially purified 1α (OH)-ase (S. Wakino et al., Gerontology 42, 67 (1996); Eva Axen, FEBS Lett. 375, 277 (1995); M. Burgos-Trinidad, R. Ismail, R. A. Ettinger, J. M. Prahl, H. F. DeLuca, J. Biol. Chem. 267, 3498 (1992); M. Warner et al., J. Biol. Chem. 257, 12995 (1982)).

Example 2

Detection of in vivo activity of CYP1AD

To confirm that CYP1AD has ability to activate the transactivating function of VDR by converting 25(OH)D_3 into active vitamin D *in vivo*, COS-1 cells were co-transformed

with 0.5 μg of GAL4-VDR(DEF) expression vector, 1 μg of 17M2-G-CAT (S. Kato et al., Science 270, 1491 (1995)), 0.5 μg each of ADX expression vector and ADR expression vector (T. Sakaki, S. Kominami, K. Hayashi, M. Akiyoshi-Shibata, Y. Yabusaki, J. Biol. Chem. 271, 26209 (1996); F. J. Dilworth et al., J. Biol. Chem. 270, 16766 (1995)), and 1 μg of CYP1AD expression vector, in the presence of $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$. A representative CAT assay is shown at the bottom panel of Figure 7. The relative CAT activities are shown at the top panel of Figure 7, as the average and SEM of three independent experiments. $25(OH)D_3$ activated the CAT reporter gene when CYP1AD was expressed, while only $1\alpha,25(OH)_2D_3$ activated the reporter gene without using CYP1AD expression vector. However, $25(OH)D_3$ did not significantly activate the reporter gene in the absence of ADX or ADR. These results strongly suggest that CYP1AD is 1α (OH)-ase, which converts $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$.

Example 3

Chemical analysis of CYP1AD products

To chemically determine the enzyme product of CYP1AD, normal phase HPLC and reversed phase HPLC were performed (E. B. Mawer et al., J. Clin. Endocrinol. Metab. 79, 554 (1994); H. Fujii et al., EMBO J., in press (1997)). The cells ($5x10^6$) transformed with ADR expression vector, ADX expression vector and CYP1AD expression vector (Figure 8(b)), or the cells ($5x10^6$) not transformed (Figure 8(c)) were incubated in the presence of [^{3}H]$25(OH)D_3$ (10^5 dpm; 6.66 terabecquerel/mmol, Amersham International) at 37°C for 6 hours. The culture media were extracted with chloroform, and the extract was analyzed by normal phase HPLC using TSK-gel silica 150 column (4.6x250mm, Tosoh), with hexane/isopropanol/methanol (88:6:6) for mobile phase, at the flow rate of 1.0 ml/min. The

eluate was collected and its radioactivity was measured using a liquid scintillation counter (E. B. Mawer et al., J. Clin. Endocrinol. Metab. 79, 554 (1994); H. Fujii et al., EMBO J. in press, (1997)). The standard samples of vitamin D derivatives, namely, 1α,(OH)D_3, 25(OH)D_3, 24,25(OH)$_2D_3$, 1α,25(OH)$_2D_3$ and 1α,24,25(OH)$_3D_3$, were applied to chromatography to determine their retention time by UV absorbance at 264 nm (Figure 8(a)).

Likewise, reverse phase HPLC was performed with a column filled with Cosmasil 5C18-AR (4.6x150 mm Nacalai Tesque) at flow rate of 1.0 ml/min to confirm the existence of [^{3}H]1α,25(OH)$_2D_3$. The chromatograms of standard samples for vitamin D derivatives, and the reaction product in the presence or absence of CYP1AD, are shown in Figure 9(a), (b), and (c), respectively.

The retention times of enzyme products in normal phase HPLC and reverse phase HPLC were completely identical to that of sample, 1α,25(OH)$_2D_3$ standard. The results indicate that the cDNA of CYP1AD encodes mouse 1α (OH)-ase, which hydroxylates 25(OH)D_3 to 1α,25(OH)$_2D_3$.

Example 4

Analysis of tissue distribution of CYP1AD transcripts

The tissue distribution of CYP1AD transcripts in 7-week-old normal and VDR-/- mice was examined. Poly(A)$^+$ RNA was extracted from brain, lung, heart, liver, spleen, kidney, small intestine, skeletal muscle, skin, and bone, and analyzed by northern blot technique using cDNA of CYP1AD and β-actin as probes (K. Takeyama et al., Biochem. Biophys. Res. Commun. 222, 395 (1996); H. Mano et al., J. Biol. Chem. 269, 1591 (1994)). As the result, the transcript of CYP1AD was detected as a single band in the kidney. The size of the transcript (2.4 kbp) is identical to that of cloned cDNA (Figure 10). Except for kidney,

in, 1α (OH)-ase activity has been reported in other tissues than kidney (A. W. Norman, J. Roth, L. Orchi, Endocr. Rev. 3, 331 (1982); H. F. DeLuca, Adv. Exp. Med. Biol. 196, 361 (1986); M. R. Walters, Endocr. Rev 13, 719 (1992); G. A. Howard, R. T. Turner, D. J. Sherrard, D. J. Baylink, J. Biol. Chem. 256, 7738 (1981); T. K. Gray, G. E. Lester, R. S. Lorenc, Science 204, 1311 (1979)). However, the transcript of 1α (OH)-ase was not detected in tissues other than kidney in this experiment.

The northern blot analysis of the expression of the CYP1AD gene and the 24(OH)-ase (CYP24) gene was performed in 3- and 7-week-old VDR+/+, VDR+/-, and VDR-/- mice, with (+) or without (-) administration of excess 1α,25$(OH)_2D_3$ (50 ng/mouse). A representative northern blot analysis is shown in Figure 11. The relative amount of the hydroxylase gene standardized with the β-actin gene transcripts was measured in at least 5 mice for each group (Figure 12). Interestingly, the marked induction of the gene was seen in VDR-/- mice (2.5 and 50 times in 3- and 7-week-old mice, respectively)(Figure 11, 12). In VDR+/+ mice and VDR+/- mice, the administration of 1α,25$(OH)_2D_3$ significantly inhibited expression of the 1α (OH)-ase gene, whereas the inhibition did not occurred in 3- and 7-week-old VDR-/- mice. Therefore, the overexpression of 1α (OH)-ase appears to cause raise in the serum level of 1α,25$(OH)_2D_3$ in 7-week-old VDR-/- mice compared with the normal level (Figure 2). Considering these results, it can be considered that ligand-bound VDR is involved in the negative regulation of the 1α (OH)-ase gene expression by 1α,25$(OH)_2D_3$. In VDR-/- mice, the expression of the 24(OH)-ase gene was decreased to the undetectable level, and the reaction against 1α,25$(OH)_2D_3$ was not seen (Figure 11, 12). The 24(OH)-ase gene converts 25$(OH)D_3$ to 24,25$(OH)_2D_3$, which is an inactive form of vitamin D, and its gene expression is positively regulated by 1α,25$(OH)_2D_3$. These results confirmed that the ligand-bound VDR is involved in the gene expression induced by 1α,25$(OH)_2D_3$ through vitamin D

responsive element in the promoter of the 24(OH)-ase gene (C. Zierold, H. M. Darwish, H. F. DeLuca, J. Biol. Chem. 270, 1675 (1995); Y. Ohyama et al., J. Biol. Chem. 269, 10545 (1994)). Therefore, the ligand-bound VDR adversely regulates the expression of 1α (OH)-ase and 24(OH)-ase genes by $1\alpha,25(OH)_2D_3$.

Example 5

Isolation of human gene encoding an enzyme that hydroxylates the 1 position of vitamin D

A normal human kidney cDNA library was prepared by extracting poly(A) RNA from normal human kidney tissue using the SacII(500bp)-Eco-RI(1200bp) fragment of mouse 1α (OH)-ase as a probe and inserting the RNA into λ-ZAPII. A human gene encoding the enzyme that hydroxylates 1α position of vitamin D was obtained by screening the library prepared above by plaque hybridization method. The nucleotide sequence of the isolated gene is shown in SEQ ID NO: 4, and the putative amino acid sequence is shown in SEQ ID NO: 2.

Industrial Applicability

The present invention provides a method for screening genes encoding polypeptides capable of converting a ligand precursor into a ligand, and a method for determining whether or not a test gene encodes a polypeptide that converts a ligand precursor into a ligand. The method of the present invention, unlike the existing expression cloning method, advantageously utilizes the nature of nuclear receptors that regulate transcription by being bound by a ligand. Since a desired gene can be detected by the reporter activity, the method of the invention enables simply and efficiently detecting and isolating a gene even if it

encodes a polypeptide that is expressed at a low level. The present invention also provides a polypeptide that converts a ligand precursor into a ligand, namely, a polypeptide that converts an inactive form of vitamin D_3 into its active form and a gene encoding it, which are obtained by the screening method as described above. The polypeptide and gene of the present invention can be used for treating and/or preventing defects in 1α (OH)-ase or renal failure. The polypeptide of the present invention can also be used to produce active vitamin D derivatives, namely, hydroxylate 1α position of vitamin D or its derivatives without a hydroxyl group at 1α position. The antibodies against the polypeptide of the present invention can be used to purify the polypeptide of the present invention, and to treat vitamin D excessiveness, granulomatous diseases, lymphoma, and the like.

In addition, the present invention provides a method for screening ligands that bind to nuclear receptors, and a method for determining whether or not a test compound is a ligand of the nuclear receptor. The method also takes advantage of the nature of nuclear receptors and uses the reporter activity for the detection. These methods are thus simple and efficient as well as the method described above. For example, the method is useful in searching ligands for orphan receptors, for which ligands are unknown.

Furthermore, the present invention provides a method for screening genes encoding polypeptides capable of converting an inactive form of transcriptional regulatory factor into an active form, based on the screening method described above. This method enables easily isolating genes that encode polypeptides capable of converting an inactive form of various transcriptional regulatory factors into the active form by detecting the reporter activity.

What is claimed is:

1. A cell comprising a vector carrying a gene encoding a nuclear receptor and a vector carrying the binding sequence of the nuclear receptor and a reporter gene located downstream of said binding sequence

2. The cell of claim 1, wherein the nuclear receptor is a vitamin D receptor.

3. A cell comprising a vector carrying a gene encoding a fusion polypeptide comprising DNA binding domain of a nuclear receptor and ligand-binding domain of a nuclear receptor, and a vector carrying the binding sequence of the DNA binding domain of the nuclear receptor and a reporter gene located downstream of the binding sequence.

4. The cell of claim 3, wherein the DNA binding domain of the nuclear receptor is originated from GAL4.

5. The cell of claim 3, wherein the ligand-binding domain of the nuclear receptor is originated from vitamin D receptor.

6. A method for screening a ligand that binds to a nuclear receptor, the method comprising

(A) contacting a test compound with the cell of claim 1,

(B) detecting the reporter activity, and

(C) selecting the test compound which elicited the reporter activity in the cell.

7. A method for determining whether or not a test compound is a ligand that binds to a nuclear receptor, the method comprising,

(A) contacting a test compound with the cell of claim 1, and

(B) detecting the reporter activity.

8. A method for screening a gene encoding a polypeptide that converts a ligand precursor into a ligand, the method comprising

(A) introducing a test gene into the cell of claim 1,

(B) contacting a ligand precursor to the cell into which the test gene is introduced,

(C) detecting the reporter activity, and

(D) isolating the test gene from the cell which showed the reporter activity.

9. A method for determining whether or not a test gene encoding a polypeptide that converts a ligand precursor into a ligand, the method comprising

(A) introducing a test gene into the cell of claim 1,

(B) contacting a ligand precursor to the cell into which the test gene is introduced, and

(C) detecting the reporter activity.

10. A method for screening a gene encoding a polypeptide that converts an inactive form of vitamin D_3 into an active form, the method comprising

(A) introducing a test gene into the cell of claim 2,

(B) contacting an inactive form of vitamin D_3 to the cell into which the test gene is introduced,

(C) detecting the reporter activity, and

(D) isolating the test gene from the cell that shows the reporter activity.

11. A method for determining whether or not a test gene encodes a polypeptide that converts an inactive form of vitamin D_3 into an active form, the method comprising

(A) introducing a test gene into the cell of claim 2,

(B) contacting an inactive form of vitamin D_3 with the cell into which the test gene is introduced, and

(C) detecting the reporter activity.

12. A ligand that binds to a nuclear receptor, which is obtainable by the method of claim 6.

13. A gene encoding a polypeptide that converts a ligand precursor into a ligand, which is obtainable by the method of claim 8.

14. A gene encoding a polypeptide that converts an inactive form of vitamin D_3 into an active form, which is obtainable by the method of claim 10.

15. A polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or its derivative comprising said sequence in which one or more amino acids are substituted,

deleted, or added, and having activity to convert an inactive form of vitamin D_3 into an active form.

16. A polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or its derivative comprising said sequence in which one or more amino acids are substituted, deleted, or added, and having activity to convert an inactive form of vitamin D_3 into an active form.

17. A polypeptide encoded by a DNA that hybridizes with a DNA having the nucleotide sequence of SEQ ID NO: 3, wherein the polypeptide has activity to convert an inactive form of vitamin D_3 into an active form.

18. A polypeptide encoded by a DNA that hybridizes with the nucleotide sequence of SEQ ID NO: 4, wherein the polypeptide has activity to convert an inactive form of vitamin D_3 into an active form.

19. A DNA encoding the polypeptide of claim 15.

20. A DNA hybridizing with a DNA having the nucleotide sequence of SEQ ID NO: 3 and encoding a polypeptide having activity to convert an inactive form of vitamin D_3 into an active form.

21. A DNA hybridizing with a DNA having the nucleotide sequence of SEQ ID NO: 4 and encoding a polypeptide having activity to convert an inactive form of vitamin D_3 into an active form.

22. A vector comprising the DNA of claim 20.

23. A transformant expressively retaining the DNA of claim 20.

24. A method for producing polypeptide, the method comprising culturing the transformant of claim 23.

25. An antibody that binds to the polypeptide of claim 15.

26. A method for screening a gene encoding a polypeptide that converts an inactive form of transcriptional regulatory factor into an active form, the method comprising

(A) introducing a test gene into cells into which a vector comprising a gene encoding an inactive form of transcriptional regulatory factor and a vector comprising the binding sequence of said inactive transcriptional regulatory factor and a reporter gene located downstream thereof are introduced,

(B) detecting the reporter activity, and

(C) isolating the test gene from the cells showing the reporter activity.

27. The method of claim 26, wherein the inactive transcriptional regulatory factor is a complex of non-phosphorylated NFκB and IκB, non-phosphorylated HSTF, or non-phosphorylated AP1.

GENE SCREENING METHOD USING NUCLEAR RECEPTOR

Abstract of the Disclosure

A system in which a ligand is formed by the expression of a polypeptide that converts a ligand precursor into a ligand, and the ligand thus formed binds to a nuclear receptor to thereby induce the expression of a reporter gene located downstream of the target sequence is constructed. Searching a gene library using this system can isolate a gene encoding a polypeptide capable of converting a ligand precursor into a ligand. This system, which takes the advantage of the transcriptional regulatory function of a nuclear receptor, enables screening a ligand that binds to a nuclear receptor and to examine whether or not a test compound is a ligand that binds to the nuclear receptor, and also screening genes that encode polypeptides capable of converting an inactive form of a wide range of transcriptional regulatory factors into an active form.

Figure 1

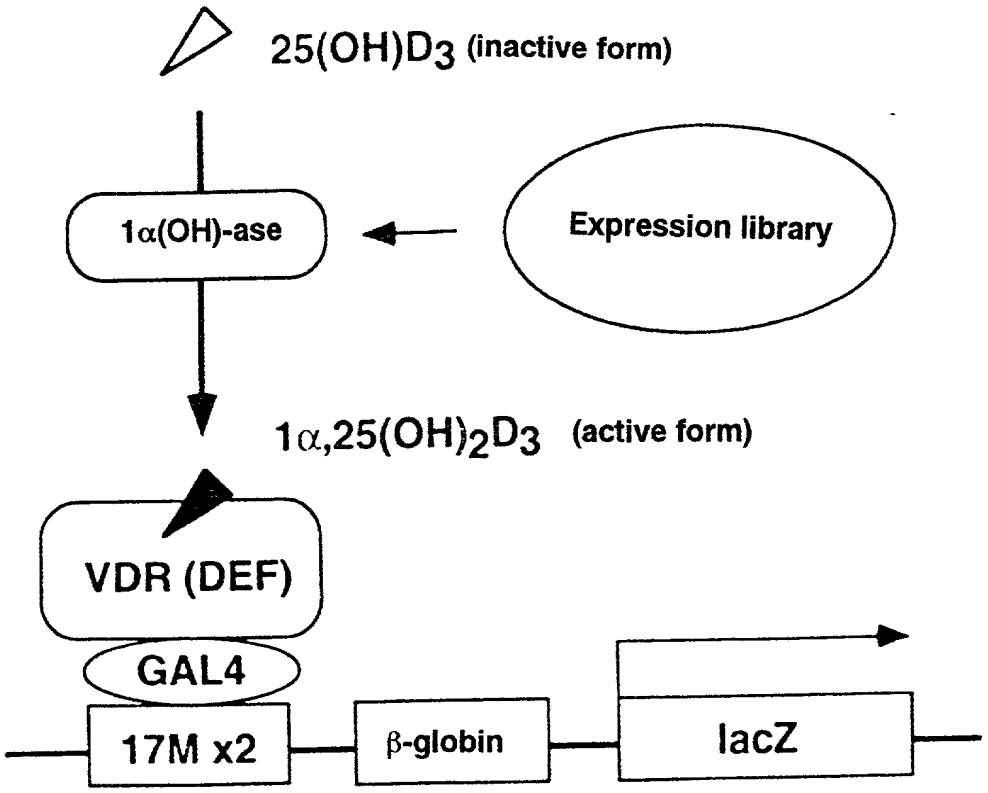
25(OH)D3 (inactive form)
1α(OH)-ase
Expression library
1α,25(OH)2D3 (active form)
VDR (DEF)
GAL4
17M x2
β-globin
lacZ

Figure 2

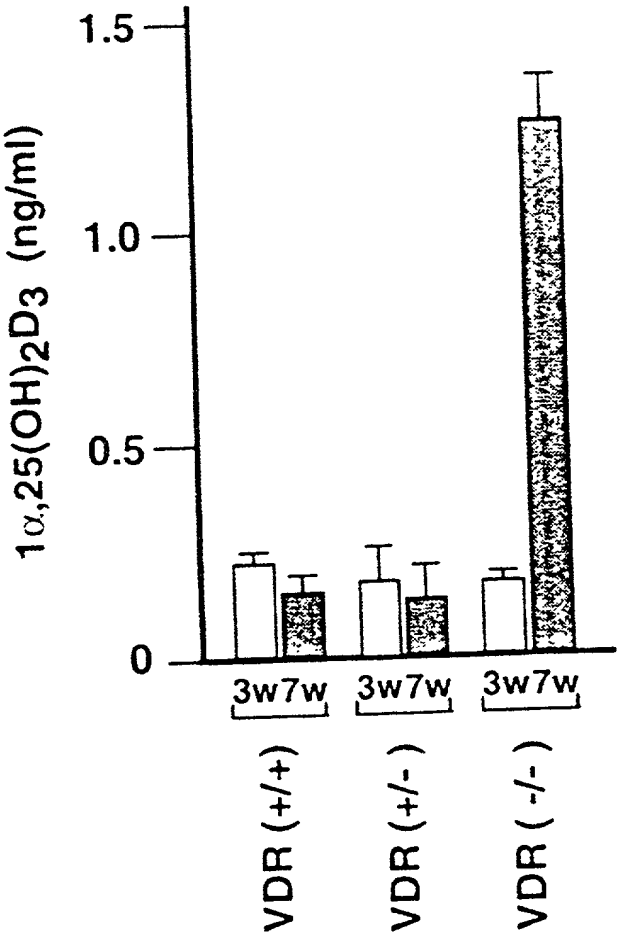

Figure 3

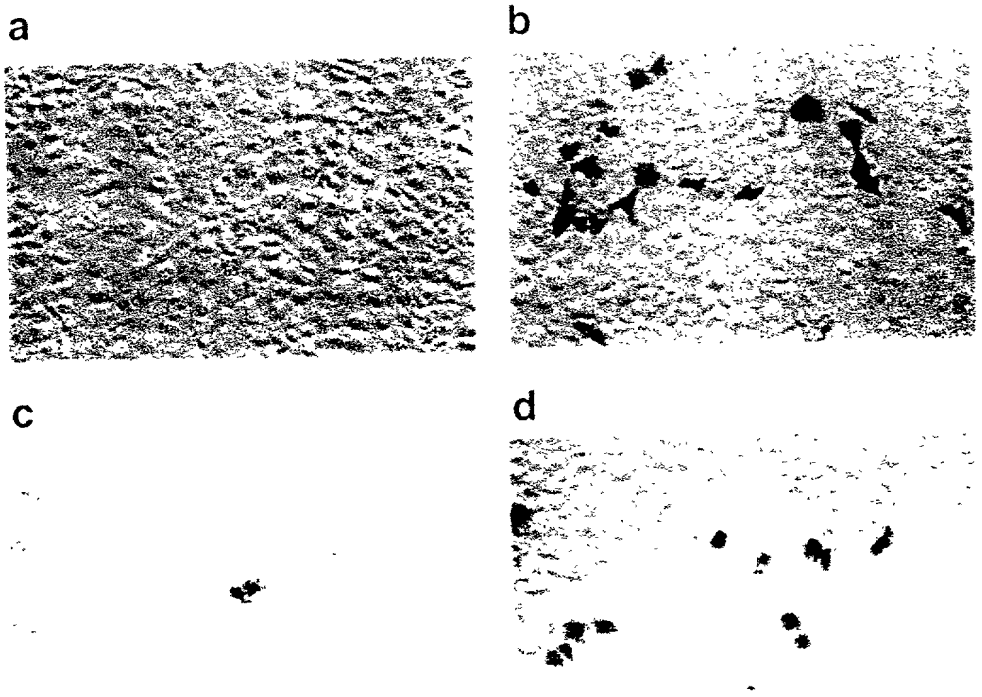

Figure 4

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 41  PGPSTLSFLAELFCKGGLSRLHELQVHGAARYGPIWSGSF  80
 81  GTLRTVYVADPTLVEQLLRQESHCPERCSFSSWAEHRRRH 120
121  QRACGLLTADGEEWQRLRSLLAPLLLRPQAAAGYAGTLDN 160
161  VVRDLVRRLRRQRGRGSGLPGLVLDVAGEFYKFGLESIGA 200
201  VLLGSRLGCLEAEVPPDTETFIHAVGSVFVSTLLTMAMPN 240
241  WLHHLIPGPWARLCRDWDQMFAFAQRHVELREGEAAMRNQ 280
281  GKPEEDMPSGHHLTHFLFREKVSVQSIVGNVTELLLAGVD 320
321  TVSNTLSWTLYELSRHPDVQTALHSEITAGTRGSCAHPHG 360
361  TALSQLPLLKAVIKEVLRLYPVVPGNSRVPDRDIRVGNYV 400
401  IPQDTLVSLCHYATSRDPTQFPDPNSFNPARWLGEGPTPH 440
441  PFASLPFGFGKRSCIGRRLAELELQMALSQILTHFEVLPE 480
481  PGALPIKPMTRTVLVPERSINLQFVDR*
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Figure 5

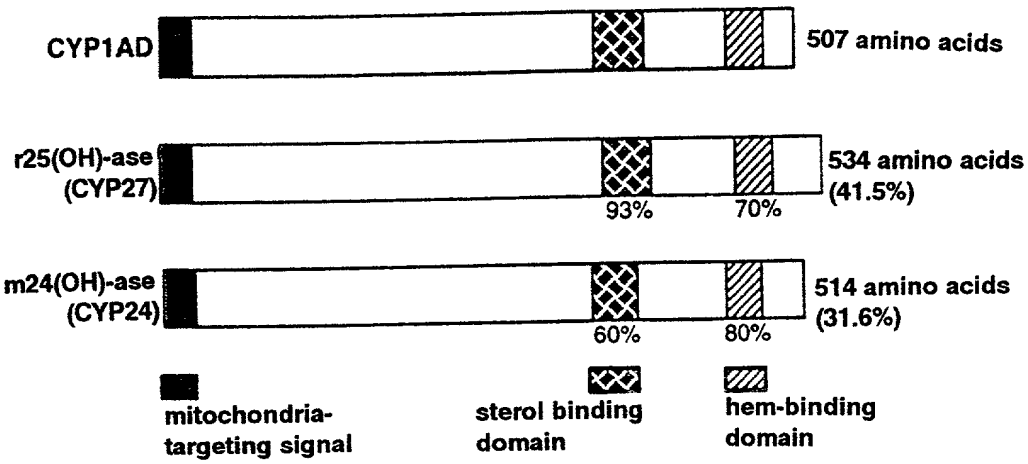

CYP1AD
507 amino acids
r25(OH)-ase
(CYP27)
534 amino acids
(41.5%)
93%
70%
m24(OH)-ase
(CYP24)
514 amino acids
(31.6%)
60%
80%
mitochondria-
targeting signal
sterol binding
domain
hem-binding
domain

Figure 6

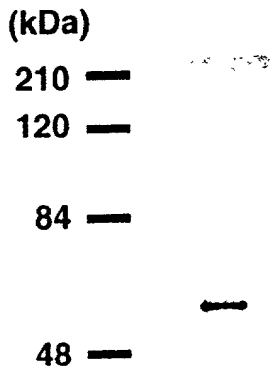


Figure 7

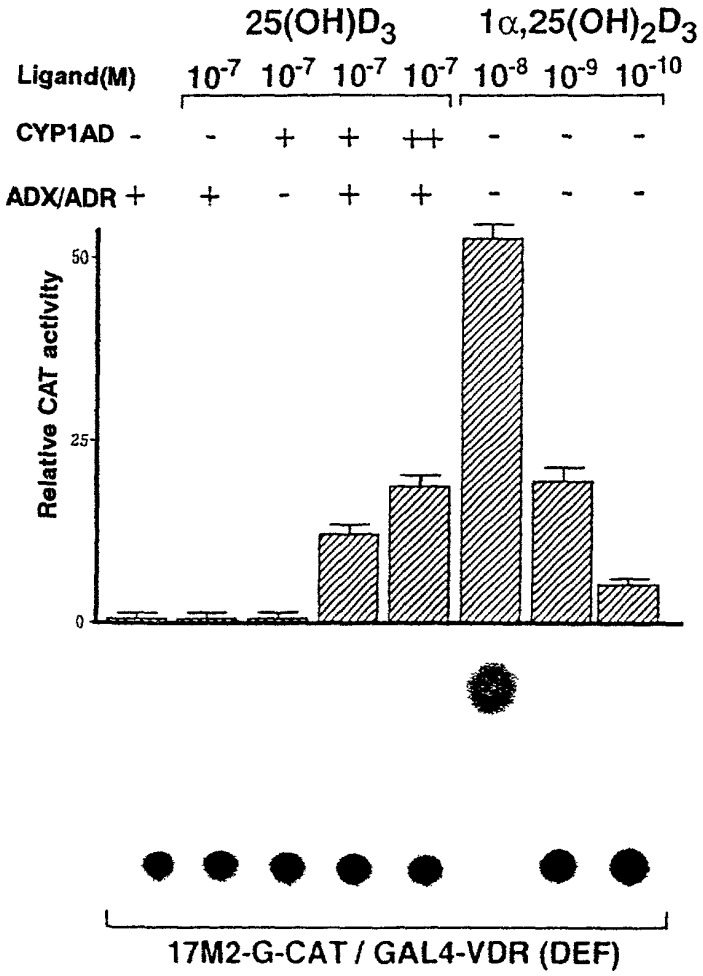

25(OH)D3
1α,25(OH)2D3
Ligand(M)
10-7 10-7 10-7 10-7 10-8 10-9 10-10
CYP1AD - - + + ++ - - -
ADX/ADR + + - + + - - -
Relative CAT activity
50
25
0
17M2-G-CAT / GAL4-VDR (DEF)

Figure 8

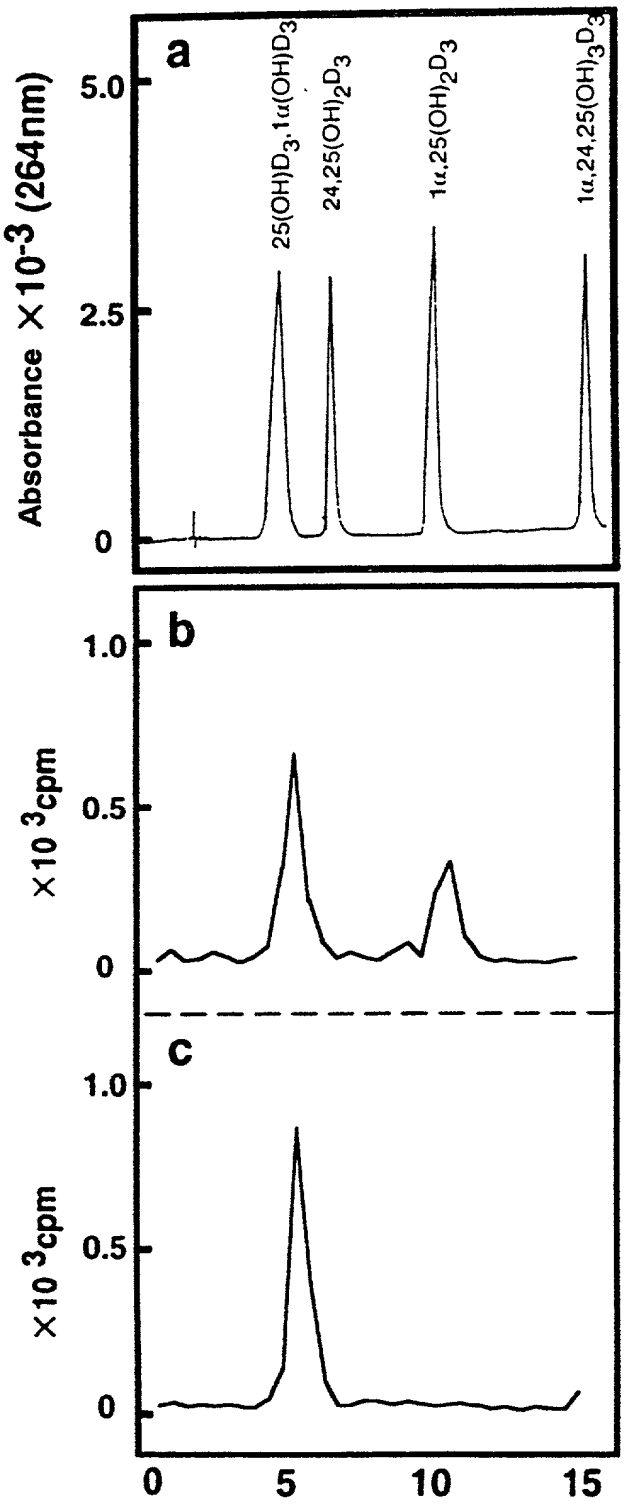

a
b
c
Absorbance ×10-3 (264nm)
×10 3cpm
×10 3cpm
25(OH)D3,1α(OH)D3
24,25(OH)2D3
1α,25(OH)2D3
1α,24,25(OH)3D3
5.0
2.5
0
1.0
0.5
0
1.0
0.5
0
0
5
10
15

Figure 9

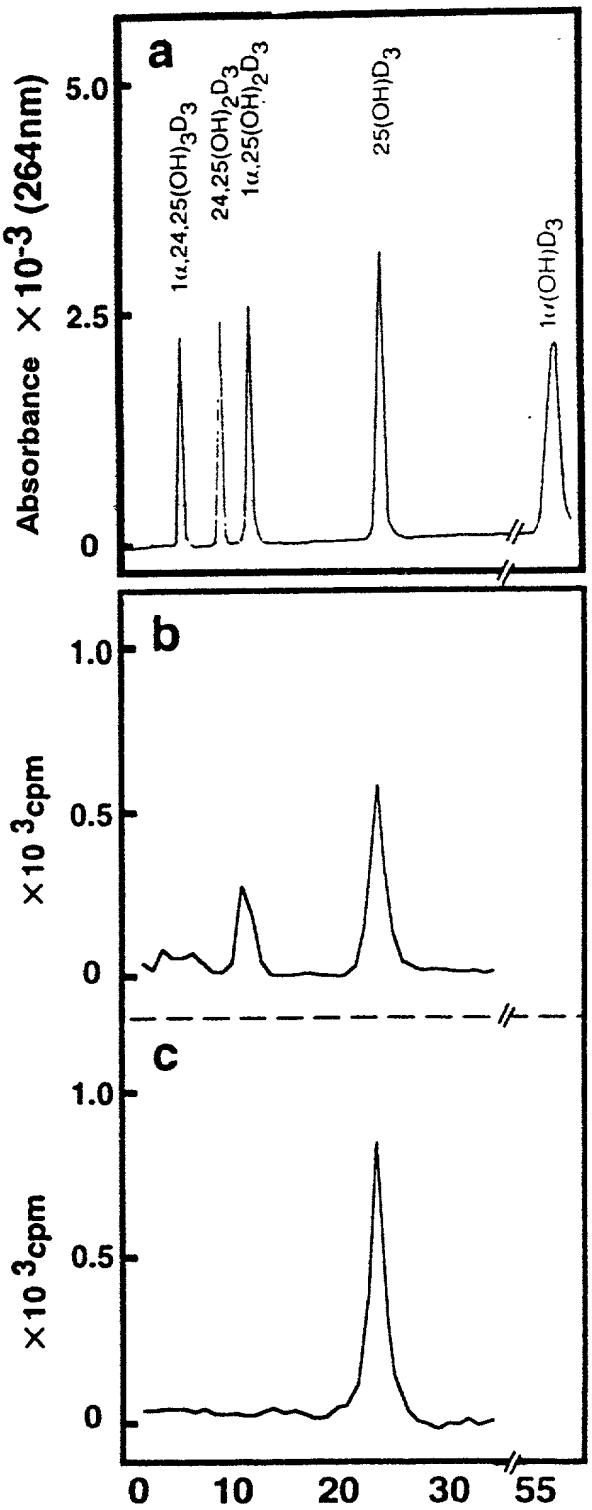

a
b
c
Absorbance ×10-3 (264nm)
5.0
2.5
0
1α,24,25(OH)3D3
24,25(OH)2D3
1α,25(OH)2D3
25(OH)D3
1α(OH)D3
1.0
0.5
0
×10 3cpm
0
10
20
30
55

Figure 10

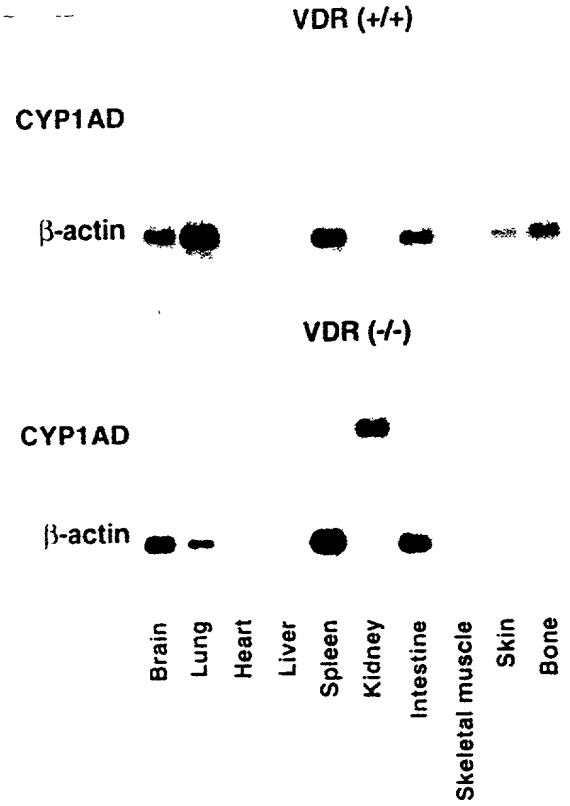

VDR (+/+)
CYP1AD
β-actin
VDR (-/-)
CYP1AD
β-actin
Brain
Lung
Heart
Liver
Spleen
Kidney
Intestine
Skeletal muscle
Skin
Bone

Figure 11

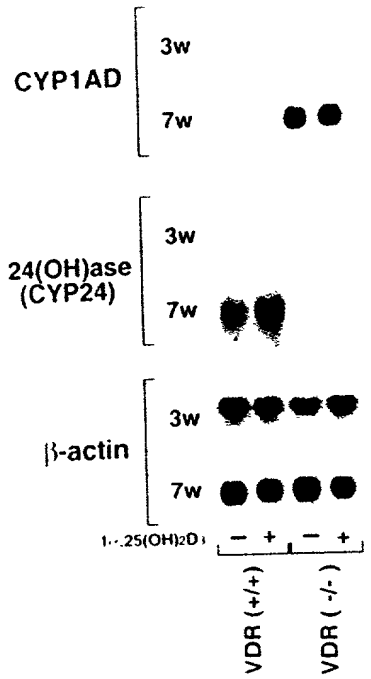
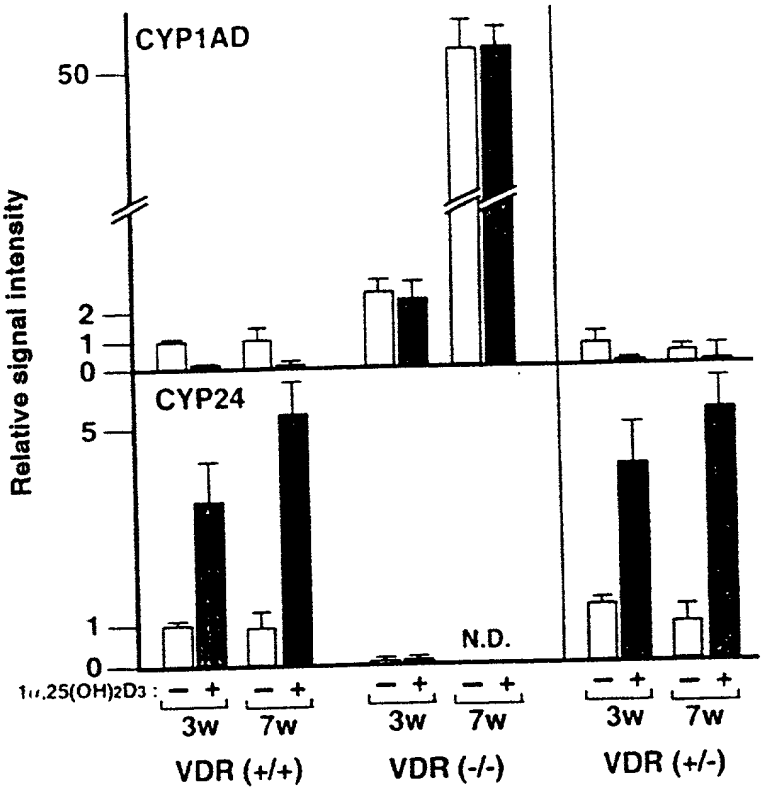

CYP1AD
3w
7w
24(OH)ase
(CYP24)
3w
7w
β-actin
3w
7w
1-.25(OH)2D3
– + – +
VDR (+/+)
VDR (-/-)

Figure 12


CYP1AD
50
2
1
0
CYP24
5
1
0
Relative signal intensity
N.D.
1α,25(OH)2D3 : − + − + − + − + − + − +
3w 7w 3w 7w 3w 7w
VDR (+/+)
VDR (-/-)
VDR (+/-)

Attorney's Docket No.: 06501-054001
Client's Ref. No.: C1-901PCT-US

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled GENE SCREENING METHOD USING NUCLEAR RECEPTOR, the specification of which:

[X] is attached hereto.
[] was filed on _____ as Application Serial No. _ and was amended on _____.
[] was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Serial No.	Filing Date	Status
PCT/JP98/03280	July 22, 1998	Pending

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
Japan	9/212624	July 22, 1997	[X] Yes [] No
			[X] Yes [] No

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Sequence Listing

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Leu Gly Ser Arg Gly Ser Glu Ser Val Leu Arg Ser Leu Ser Asp Ile
25                  30                  35                  40
Pro Gly Pro Ser Thr Leu Ser Phe Leu Ala Glu Leu Phe Cys Lys Gly
                45                  50                  55
Gly Leu Ser Arg Leu His Glu Leu Gln Val His Gly Ala Ala Arg Tyr
            60                  65                  70
Gly Pro Ile Trp Ser Gly Ser Phe Gly Thr Leu Arg Thr Val Tyr Val
        75                  80                  85
```

```
Ala Asp Pro Thr Leu Val Glu Gln Leu Leu Arg Gln Glu Ser His Cys
    90                  95                 100
Pro Glu Arg Cys Ser Phe Ser Ser Trp Ala Glu His Arg Arg Arg His
105                 110                 115                 120
Gln Arg Ala Cys Gly Leu Leu Thr Ala Asp Gly Glu Glu Trp Gln Arg
                125                 130                 135
Leu Arg Ser Leu Leu Ala Pro Leu Leu Leu Arg Pro Gln Ala Ala Ala
            140                 145                 150
Gly Tyr Ala Gly Thr Leu Asp Asn Val Val Arg Asp Leu Val Arg Arg
        155                 160                 165
Leu Arg Arg Gln Arg Gly Arg Gly Ser Gly Leu Pro Gly Leu Val Leu
    170                 175                 180
Asp Val Ala Gly Glu Phe Tyr Lys Phe Gly Leu Glu Ser Ile Gly Ala
185                 190                 195                 200
Val Leu Leu Gly Ser Arg Leu Gly Cys Leu Glu Ala Glu Val Pro Pro
                205                 210                 215
Asp Thr Glu Thr Phe Ile His Ala Val Gly Ser Val Phe Val Ser Thr
            220                 225                 230
Leu Leu Thr Met Ala Met Pro Asn Trp Leu His His Leu Ile Pro Gly
        235                 240                 245
Pro Trp Ala Arg Leu Cys Arg Asp Trp Asp Gln Met Phe Ala Phe Ala
    250                 255                 260
Gln Arg His Val Glu Leu Arg Glu Gly Glu Ala Ala Met Arg Asn Gln
265                 270                 275                 280
Gly Lys Pro Glu Glu Asp Met Pro Ser Gly His His Leu Thr His Phe
                285                 290                 295
Leu Phe Arg Glu Lys Val Ser Val Gln Ser Ile Val Gly Asn Val Thr
            300                 305                 310
Glu Leu Leu Leu Ala Gly Val Asp Thr Val Ser Asn Thr Leu Ser Trp
        315                 320                 325
Thr Leu Tyr Glu Leu Ser Arg His Pro Asp Val Gln Thr Ala Leu His
    330                 335                 340
Ser Glu Ile Thr Ala Gly Thr Arg Gly Ser Cys Ala His Pro His Gly
345                 350                 355                 360
Thr Ala Leu Ser Gln Leu Pro Leu Leu Lys Ala Val Ile Lys Glu Val
                365                 370                 375
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Leu Arg Leu Tyr Pro Val Val Pro Gly Asn Ser Arg Val Pro Asp Arg
380 385 390
Asp Ile Arg Val Gly Asn Tyr Val Ile Pro Gln Asp Thr Leu Val Ser
395 400 405
Leu Cys His Tyr Ala Thr Ser Arg Asp Pro Thr Gln Phe Pro Asp Pro
410 415 420
Asn Ser Phe Asn Pro Ala Arg Trp Leu Gly Glu Gly Pro Thr Pro His
425 430 435 440
Pro Phe Ala Ser Leu Pro Phe Gly Phe Gly Lys Arg Ser Cys Ile Gly
445 450 455
Arg Arg Leu Ala Glu Leu Glu Leu Gln Met Ala Leu Ser Gln Ile Leu
460 465 470
Thr His Phe Glu Val Leu Pro Glu Pro Gly Ala Leu Pro Ile Lys Pro
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Val Asp Arg
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<213> Homo sapiens

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20 25 30
Ala Arg Arg Ser Leu Ala Asp Ile Pro Gly Pro Ser Thr Pro Ser Phe
35 40 45
Leu Ala Glu Leu Phe Cys Lys Gly Gly Leu Ser Arg Leu His Glu Leu
50 55 60
Gln Val Gln Gly Ala Ala His Phe Gly Pro Val Trp Leu Ala Ser Phe
65 70 75 80
Gly Thr Val Arg Thr Val Tyr Val Ala Ala Pro Ala Leu Val Glu Glu

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                85                  90                  95
Leu Leu Arg Gln Glu Gly Pro Arg Pro Glu Arg Cys Ser Phe Ser Pro
            100                 105                 110
Trp Thr Glu His Arg Arg Cys Arg Gln Arg Ala Cys Gly Leu Leu Thr
        115                 120                 125
Ala Glu Gly Glu Glu Trp Gln Arg Leu Arg Ser Leu Leu Ala Pro Leu
    130                 135                 140
Leu Leu Arg Pro Gln Ala Ala Ala Arg Tyr Ala Gly Thr Leu Asn Asn
145                 150                 155                 160
Val Val Cys Asp Leu Val Arg Arg Leu Arg Arg Gln Arg Gly Arg Gly
                165                 170                 175
Thr Gly Pro Pro Ala Leu Val Arg Asp Val Ala Gly Glu Phe Tyr Lys
            180                 185                 190
Phe Gly Leu Glu Gly Ile Ala Ala Val Leu Leu Gly Ser Arg Leu Gly
        195                 200                 205
Cys Leu Glu Ala Gln Val Pro Pro Asp Thr Glu Thr Phe Ile Arg Ala
    210                 215                 220
Val Gly Ser Val Phe Val Ser Thr Leu Leu Thr Met Ala Met Pro His
225                 230                 235                 240
Trp Leu Arg His Leu Val Pro Gly Pro Trp Gly Arg Leu Cys Arg Asp
                245                 250                 255
Trp Asp Gln Met Phe Ala Phe Ala Gln Arg His Val Glu Arg Arg Glu
            260                 265                 270
Ala Glu Ala Ala Met Arg Asn Gly Gly Gln Pro Glu Lys Asp Leu Glu
        275                 280                 285
Ser Gly Ala His Leu Thr His Phe Leu Phe Arg Glu Glu Leu Pro Ala
    290                 295                 300
Gln Ser Ile Leu Gly Asn Val Thr Glu Leu Leu Leu Ala Gly Val Asp
305                 310                 315                 320
Thr Val Ser Asn Thr Leu Ser Trp Ala Leu Tyr Glu Leu Ser Arg His
                325                 330                 335
Pro Glu Val Gln Thr Ala Leu His Ser Glu Ile Thr Ala Ala Leu Ser
            340                 345                 350
Pro Gly Ser Ser Ala Tyr Pro Ser Ala Thr Val Leu Ser Gln Leu Pro
        355                 360                 365
Leu Leu Lys Ala Val Val Lys Glu Val Leu Arg Leu Tyr Pro Val Val
```

```
    370                 375                 380
Pro Gly Asn Ser Arg Val Pro Asp Lys Asp Ile His Val Gly Asp Tyr
385                 390                 395                 400
Ile Ile Pro Lys Asn Thr Leu Val Thr Leu Cys His Tyr Ala Thr Ser
                405                 410                 415
Arg Asp Pro Ala Gln Phe Pro Glu Pro Asn Ser Phe Arg Pro Ala Arg
            420                 425                 430
Trp Leu Gly Glu Gly Pro Thr Pro His Pro Phe Ala Ser Leu Pro Phe
        435                 440                 445
Gly Phe Gly Lys Arg Ser Cys Met Gly Arg Arg Leu Ala Glu Leu Glu
    450                 455                 460
Leu Gln Met Ala Leu Ala Gln Ile Leu Thr His Phe Glu Val Gln Pro
465                 470                 475                 480
Glu Pro Gly Ala Ala Pro Val Arg Pro Lys Thr Arg Thr Val Leu Val
                485                 490                 495
Pro Glu Arg Ser Ile Asn Leu Gln Phe Leu Asp Arg
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                                1               5
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Ser Arg Val Phe His Arg Ile His Leu Pro Leu Gln Leu Asp Ala Ser
    10                  15                  20
ctg ggc tcc aga ggc agt gag tcg gtt ctc cgg agc ttg tct gac atc     149
Leu Gly Ser Arg Gly Ser Glu Ser Val Leu Arg Ser Leu Ser Asp Ile
```

```
25                  30                  35                  40
cct ggg ccc tct aca ctc agc ttc ctg gct gaa ctc ttc tgc aaa ggg      197
Pro Gly Pro Ser Thr Leu Ser Phe Leu Ala Glu Leu Phe Cys Lys Gly
                45                  50                  55
ggg ctg tcc agg ctg cat gaa ctg cag gtg cat ggc gct gcg cgg tac      245
Gly Leu Ser Arg Leu His Glu Leu Gln Val His Gly Ala Ala Arg Tyr
            60                  65                  70
ggg cca ata tgg tct ggc agc ttt ggg aca ctt cgc aca gtt tac gtt      293
Gly Pro Ile Trp Ser Gly Ser Phe Gly Thr Leu Arg Thr Val Tyr Val
        75                  80                  85
gcc gac cct aca ctt gtg gag cag ctc ctg cga caa gaa agt cac tgt      341
Ala Asp Pro Thr Leu Val Glu Gln Leu Leu Arg Gln Glu Ser His Cys
    90                  95                  100
cca gag cgc tgt agt ttc tca tca tgg gca gag cac cgt cgc cgc cac      389
Pro Glu Arg Cys Ser Phe Ser Ser Trp Ala Glu His Arg Arg Arg His
105                 110                 115                 120
cag cgt gct tgc gga ttg cta acg gcg gat ggt gaa gaa tgg cag agg      437
Gln Arg Ala Cys Gly Leu Leu Thr Ala Asp Gly Glu Glu Trp Gln Arg
                125                 130                 135
ctc cga agt ctt ctg gcc ccg ctc ctc ctc cgg cca caa gca gcc gcg      485
Leu Arg Ser Leu Leu Ala Pro Leu Leu Leu Arg Pro Gln Ala Ala Ala
            140                 145                 150
ggc tat gct gga act ctg gac aac gtg gtc cgt gac ctt gtg cga cga      533
Gly Tyr Ala Gly Thr Leu Asp Asn Val Val Arg Asp Leu Val Arg Arg
        155                 160                 165
cta agg cgc cag cgg gga cgt ggc tct ggg cta ccc ggc cta gtt ctg      581
Leu Arg Arg Gln Arg Gly Arg Gly Ser Gly Leu Pro Gly Leu Val Leu
    170                 175                 180
gac gtg gca gga gag ttt tac aaa ttt ggc cta gaa agt ata ggc gcg      629
Asp Val Ala Gly Glu Phe Tyr Lys Phe Gly Leu Glu Ser Ile Gly Ala
185                 190                 195                 200
gtg ctg ctg gga tcg cgc ctg ggc tgc cta gag gct gaa gtc cct cct      677
Val Leu Leu Gly Ser Arg Leu Gly Cys Leu Glu Ala Glu Val Pro Pro
                205                 210                 215
gac aca gaa acc ttc ata cat gca gtg ggc tca gtg ttt gtg tct aca      725
Asp Thr Glu Thr Phe Ile His Ala Val Gly Ser Val Phe Val Ser Thr
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                220                 225                 230
ctc ttg acc atg gcg atg ccc aac tgg ttg cac cac ctt ata cct gga      773
Leu Leu Thr Met Ala Met Pro Asn Trp Leu His His Leu Ile Pro Gly
        235                 240                 245
ccc tgg gcc cgc ctc tgc cga gac tgg gat cag atg ttt gcc ttt gcc      821
Pro Trp Ala Arg Leu Cys Arg Asp Trp Asp Gln Met Phe Ala Phe Ala
    250                 255                 260
cag agg cac gtg gag ctg cga gaa ggt gaa gct gcg atg agg aac cag      869
Gln Arg His Val Glu Leu Arg Glu Gly Glu Ala Ala Met Arg Asn Gln
265                 270                 275                 280
gga aag cct gag gag gat atg ccg tct ggg cat cac tta acc cac ttc      917
Gly Lys Pro Glu Glu Asp Met Pro Ser Gly His His Leu Thr His Phe
                285                 290                 295
ctt ttt cgg gaa aag gtg tct gtc cag tcc ata gtg ggg aat gtg aca      965
Leu Phe Arg Glu Lys Val Ser Val Gln Ser Ile Val Gly Asn Val Thr
            300                 305                 310
gag cta cta ctg gct gga gtg gac acg gta tcc aat acg ctc tcc tgg     1013
Glu Leu Leu Leu Ala Gly Val Asp Thr Val Ser Asn Thr Leu Ser Trp
        315                 320                 325
aca ctc tat gag ctt tcc cgg cac ccc gat gtc cag act gca ctc cac     1061
Thr Leu Tyr Glu Leu Ser Arg His Pro Asp Val Gln Thr Ala Leu His
    330                 335                 340
tct gag atc aca gct ggg acc cgt ggc tcc tgt gcc cac ccc cat ggc     1109
Ser Glu Ile Thr Ala Gly Thr Arg Gly Ser Cys Ala His Pro His Gly
345                 350                 355                 360
act gct ctg tcc cag ctg ccc ctg tta aag gct gtg atc aaa gaa gtg     1157
Thr Ala Leu Ser Gln Leu Pro Leu Leu Lys Ala Val Ile Lys Glu Val
                365                 370                 375
ttg aga ttg tac cct gtg gta cct ggg aat tcc cgt gtc cca gac aga     1205
Leu Arg Leu Tyr Pro Val Val Pro Gly Asn Ser Arg Val Pro Asp Arg
            380                 385                 390
gac atc cgt gta gga aac tat gta att ccc caa gat acg cta gtc tcc     1253
Asp Ile Arg Val Gly Asn Tyr Val Ile Pro Gln Asp Thr Leu Val Ser
        395                 400                 405
cta tgt cac tat gcc act tca agg gac ccc aca cag ttt cca gac ccc     1301
Leu Cys His Tyr Ala Thr Ser Arg Asp Pro Thr Gln Phe Pro Asp Pro
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Pro Phe Ala Ser Leu Pro Phe Gly Phe Gly Lys Arg Ser Cys Ile Gly
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aga cgc ttg gca gag ctt gag cta caa atg gct ttg tcc cag atc ttg     1445
Arg Arg Leu Ala Glu Leu Glu Leu Gln Met Ala Leu Ser Gln Ile Leu
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acc cat ttt gaa gtg cta cct gag cca ggt gct ctt cct atc aag ccc     1493
Thr His Phe Glu Val Leu Pro Glu Pro Gly Ala Leu Pro Ile Lys Pro
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atg acc cgg act gtc ctg gtc cct gag agg agc atc aat cta cag ttt     1541
Met Thr Arg Thr Val Leu Val Pro Glu Arg Ser Ile Asn Leu Gln Phe
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gta gat aga taaccattcg gaagacagcc aacatcgtct ctctcaagac             1590
Val Asp Arg
505
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Trp Ala Pro Glu Leu Gly Ala Ser Leu Gly Tyr Arg Glu Tyr His Ser
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gca cgc cgg agc ttg gca gac atc cca ggc ccc tct acg ccc agc ttt     144
Ala Arg Arg Ser Leu Ala Asp Ile Pro Gly Pro Ser Thr Pro Ser Phe
        35                  40                  45
ctg gcc gaa ctt ttc tgc aag ggg ggg ctg tcg agg cta cac gag ctg     192
Leu Ala Glu Leu Phe Cys Lys Gly Gly Leu Ser Arg Leu His Glu Leu
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Gln Val Gln Gly Ala Ala His Phe Gly Pro Val Trp Leu Ala Ser Phe
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ggg aca gtg cgc acc gtg tac gtg gct gcc cct gca ctc gtc gag gag     288
Gly Thr Val Arg Thr Val Tyr Val Ala Ala Pro Ala Leu Val Glu Glu
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ctg ctg cga cag gag gga ccc cgg ccc gag cgc tgc agc ttc tcg ccc     336
Leu Leu Arg Gln Glu Gly Pro Arg Pro Glu Arg Cys Ser Phe Ser Pro
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tgg acg gag cac cgc cgc tgc cgc cag cgg gct tgc gga ctg ctc act     384
Trp Thr Glu His Arg Arg Cys Arg Gln Arg Ala Cys Gly Leu Leu Thr
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gcg gaa ggc gaa gaa tgg caa agg ctc cgc agt ctc ctg gcc ccg ctc     432
Ala Glu Gly Glu Glu Trp Gln Arg Leu Arg Ser Leu Leu Ala Pro Leu
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ctc ctc cgg cct caa gcg gcc gcc cgc tac gcc gga acc ctg aac aac     480
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Val Val Cys Asp Leu Val Arg Arg Leu Arg Arg Gln Arg Gly Arg Gly
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Thr Gly Pro Pro Ala Leu Val Arg Asp Val Ala Gly Glu Phe Tyr Lys
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Phe Gly Leu Glu Gly Ile Ala Ala Val Leu Leu Gly Ser Arg Leu Gly
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Cys Leu Glu Ala Gln Val Pro Pro Asp Thr Glu Thr Phe Ile Arg Ala
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Trp Asp Gln Met Phe Ala Phe Ala Gln Arg His Val Glu Arg Arg Glu
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Leu Leu Lys Ala Val Val Lys Glu Val Leu Arg Leu Tyr Pro Val Val
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Pro Gly Asn Ser Arg Val Pro Asp Lys Asp Ile His Val Gly Asp Tyr
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Ile Ile Pro Lys Asn Thr Leu Val Thr Leu Cys His Tyr Ala Thr Ser
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Arg Asp Pro Ala Gln Phe Pro Glu Pro Asn Ser Phe Arg Pro Ala Arg
            420                 425                 430
tgg ctg ggg gag ggt ccc acc ccc cac cca ttt gca tct ctt ccc ttt    1344
Trp Leu Gly Glu Gly Pro Thr Pro His Pro Phe Ala Ser Leu Pro Phe
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ggc ttt ggc aag cgc agc tgt atg ggg aga cgc ctg gca gag ctt gaa    1392
Gly Phe Gly Lys Arg Ser Cys Met Gly Arg Arg Leu Ala Glu Leu Glu
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Leu Gln Met Ala Leu Ala Gln Ile Leu Thr His Phe Glu Val Gln Pro
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